

Green Pretreatment Using Deep Eutectic Solvents to Enhance Lipid Recovery from *Chlorella vulgaris*

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Abstract

Biodiesel from microalgae is considered a promising renewable fuel, but the thick cell wall of *Chlorella vulgaris* makes lipid recovery difficult. This research used green deep eutectic solvents (DESs) as a pretreatment to improve the lipid extraction performance. Two choline chloride-based DESs, namely choline chloride–acetic acid (CCA) and choline chloride–urea (CCU), were tested at several concentrations before the biomass was extracted using ultrasound-assisted extraction with the Folch solvent system (chloroform:methanol, 2:1 v/v). Lipid content was determined using the sulphophosphovanillin (SPV) method. The result showed that both DESs increased lipid recovery compared to extraction without pretreatment, although the performance was strongly affected by the solvent composition. The highest lipid yield for CCA was obtained at 85% concentration, which was 2,105.18 μL , or 36.1% higher than the control (1,546.47 μL). Meanwhile, 70% CCU gave the best result of 3,516.10 μL , an increase of 127.4% compared to the untreated biomass. This increase in extraction efficiency was caused by the disruption of the microalgal cell wall through hydrogen-bond interactions and better solvent penetration during ultrasonic cavitation. From the comparison, CCU-based DES was more effective than CCA under the tested conditions, showing that CCU is more suitable as a green pretreatment for lipid recovery from *Chlorella vulgaris*.

Keywords: *Microalgae, Deep Eutectic Solvents, Lipid, Renewable Fuel, Extraction*

INTRODUCTION

Fossil fuel reserves continue to decrease, while the need to reduce greenhouse gas emissions is increasing. These conditions have encouraged the development of renewable fuels, including biodiesel from microalgae (Kanwal et al., 2025). Microalgae are considered a promising third-generation feedstock because they grow rapidly, can contain high lipid fractions, and can be cultivated without directly competing with food crops (Liu et al., 2022). *Chlorella vulgaris* is one of the important candidates because its cultivation is relatively easy and its biomass can contain a considerable lipid fraction (khalaji, 2022).

However, lipid recovery from *C. vulgaris* remains difficult because the cell wall is thick, rigid, and structurally complex. This structure creates a mass-transfer resistance that limits the contact between intracellular lipid and the extraction solvent (Matchim Kamdem et al., 2023). Conventional extraction methods can provide good lipid recovery, but they commonly require large amounts of organic solvent and may involve toxic and volatile solvents such as chloroform and methanol. Mechanical cell-disruption methods can also increase energy consumption. Therefore, a pretreatment that can weaken the cell wall under relatively mild conditions is needed.

Deep eutectic solvents (DES) have received increasing attention as an alternative pretreatment medium. DES is generally formed from a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), commonly using choline chloride with urea, glycerol, or organic acids. The interaction between the components produces a mixture with a melting point much lower than

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those of the individual components (Abbott et al., 2003). DES is attractive because it can be prepared by simple mixing and heating, has low vapor pressure, and can show good biodegradability and recyclability depending on its composition (Zhang et al., 2025a). However, the term green solvent should not automatically be interpreted as non-toxic. DES performance and environmental impact depend on its components, concentration, physicochemical properties, and recyclability (Vieira Sanches et al., 2023).

Several studies have shown that DES can improve the disruption of microalgal biomass. Danilović et al. (2021) reported that choline chloride-based DES combined with sonication increased lipid extraction from *Chlorella* sp. Dardavila et al. (2023) also showed that DES can be used to extract several valuable compounds from *C. vulgaris*. The mechanism is generally related to hydrogen-bond interactions between DES components and functional groups in the biomass, which can weaken the cell-wall structure and increase permeability. Ultrasound-assisted extraction (UAE) can further improve this process through acoustic cavitation, which promotes cell disruption and mass transfer.

Although DES pretreatment has been studied for microalgal extraction, information about the lipid extraction behavior of *C. vulgaris* after DES pretreatment is still limited. Extraction kinetics are important because the extraction rate, saturation yield, and mass-transfer behavior are needed for process optimization and scale-up (Motlagh et al., 2022). Therefore, this study investigates the optimum condition of lipid recovery from *Chlorella vulgaris* after pretreatment using two choline chloride-based deep eutectic solvents, namely choline chloride-acetic acid (CCA) and choline chloride-urea (CCU). The results provide a better understanding of the effect of DES pretreatment to the efficiency biodiesel production from microalgae. Furthermore, this research offers significant benefits; the use of eco-friendly Deep Eutectic Solvents (DES) for pretreatment, combined with the Ultrasound-Assisted Extraction (UAE) method, will have a substantial impact on lipid extraction from the microalga *Chlorella vulgaris*. The success of this study will contribute to achieving SDG 7 (Affordable and Clean Energy) which targets increased renewable energy utilization as well as SDG 14 (Life Below Water) by alleviating ecological pressures caused by resource exploitation and marine environmental pollution.

LITERATURE REVIEW

Chlorella vulgaris

Chlorella vulgaris has been widely studied as a feedstock for biodiesel and other value-added products because it can accumulate lipids without requiring large areas of arable land. Its lipid fraction contains saturated and unsaturated fatty acids that can be converted into biodiesel. However, the actual lipid content depends on cultivation conditions, strain, nutrient availability, and environmental stress (Loh et al., 2021; Maurício et al., 2023).

The lipid fraction of *C. vulgaris* contains several lipid classes, including triacylglycerols, phospholipids, glycolipids, and free fatty acids. Therefore, extraction is not only a matter of lipid solubility. It involves cell disruption, lipid release, diffusion, partitioning, and solubilization. Solvent polarity can affect the recovery of different lipid classes (Couto et al., 2022).

The cell wall is one of the main limitations in lipid recovery. Weber et al. (2022) reported that the cell wall of *C. vulgaris* contains neutral sugars, uronic acids, glucosamine-containing polymers, and rigid structural components related to cellulose/starch-like materials. Their analysis reported approximately 20-25 wt% neutral sugars, 15-20 wt% uronic acids, 7-17 wt% glucosamine, and 6-10 wt% protein in the cell-wall fraction. This structure explains why extending extraction time alone may not be enough. If the cell wall remains intact, the solvent still faces a high mass-transfer resistance before reaching the intracellular lipid.

Conventional and Intensified Lipid Extraction

Soxhlet, Folch, and Bligh-Dyer methods are commonly used as reference procedures for microalgal lipid extraction. They can provide relatively high recovery but have limitations related to solvent consumption, extraction time, solvent toxicity, volatility, and solvent recovery (Zhou et al., 2022). Innovative methods such as UAE, microwave-assisted extraction, supercritical-fluid extraction, and pressurized-liquid extraction can improve extraction efficiency or shorten processing time. Foerster et al. (2023) showed that extraction performance is strongly influenced by the combination of cell disruption and solvent extraction. This means that pretreatment and solvent selection should be considered as one integrated process.

Deep Eutectic Solvents as Pretreatment Media

The physicochemical properties of DES depend on the HBA:HBD ratio, water content, temperature, and molecular interactions. These factors influence viscosity, polarity, conductivity, density, and mass transfer (Yeow et al., 2024). Viscosity is particularly important because a highly viscous DES can reduce molecular mobility and solvent penetration.

The proposed mechanism of DES pretreatment can be described as DES penetration→disruption or reorganization of hydrogen bonds → weakening of the cell-wall structure→ increased permeability→better solvent penetration→lipid release. DES components can interact with hydroxyl, carboxyl, amino, and other polar groups in the biomass and compete with the original intermolecular interactions. Huang et al. (2022) reported a strong increase in lipid extraction efficiency after DES-assisted treatment, supporting the role of DES in changing biomass interactions and facilitating lipid diffusion.

Water content also needs to be considered. Adding water can reduce DES viscosity and improve diffusion, but excessive water can weaken the hydrogen-bond interactions that give DES its specific properties. Therefore, an optimum condition is expected between a system that is too viscous and a system that is too diluted.

Solvent recovery is another important issue for scale-up. Most DES have very low vapor pressure, so they cannot be recovered simply by evaporation like conventional volatile solvents. Lo et al. (2024) showed that polar antisolvents can be used for lipid recovery from DES and that the DES can also be regenerated. Therefore, the sustainability of DES should be evaluated not only from extraction performance but also from solvent consumption, recovery, reuse, and waste generation.

Lipid Extraction

Zhang et al. (2025a) studied lipid extraction from DES-pretreated *Chlorella pyrenoidosa* using methanol:ethyl acetate (2:1). Although the species was different from *C. vulgaris*, the study is relevant because it combined DES pretreatment, solid-liquid extraction, temperature variation, and kinetic modeling. Lipid concentration increased rapidly at the beginning and then became slower. The maximum lipid concentration from dry biomass was 95 mg g⁻¹ at 70°C after 90 minutes. This behavior indicates that extraction is commonly controlled by a combination of rapid release and slower diffusion from less accessible regions of the biomass.

RESEARCH METHODOLOGY

Equipment and Materials

The equipment included an analytical balance, beakers, magnetic stirrer and stir bars, oven, mortar and pestle, ultrasonic cleaner bath, centrifuge, Erlenmeyer flasks, heater/hot plate, reflux condenser, three-neck flask, thermometer, UV-Vis spectrophotometer, and micropipettes. The materials were *Chlorella vulgaris* microalgae (100mesh), choline chloride (>98%), glacial acetic acid (99%), technical-grade urea, chloroform, methanol (99%), ethanol (99%), analytical-grade

vanillin, phosphoric acid (85%), sulfuric acid (98%), distilled water, and canola oil.

DES Pretreatment and Ultrasound-Assisted Lipid Extraction

CCA and CCU were prepared by mixing choline chloride with acetic acid or urea at a molar ratio of 1:2. The mixture was heated at 80°C with stirring until a homogeneous and transparent solution was obtained. The DES was then diluted to 40, 55, 70, 85, and 100% (v/v).

For pretreatment, 10 g of dry *C. vulgaris* biomass was mixed with DES at a biomass-to-DES ratio of 1:20 (w/v). CCA pretreatment was conducted at 50°C, while CCU pretreatment was conducted at 80°C. Both treatments were performed for 60 minutes under magnetic stirring. After pretreatment, the mixture was centrifuged to separate the biomass from the DES solution, and the biomass was dried again before extraction to reduce residual DES.

Lipid extraction was performed using UAE with a reflux system. Pretreated and untreated biomass were extracted using chloroform:methanol (2:1, v/v) with a total solvent volume of 300 mL. Extraction was conducted at 60°C for 100 minutes using an ultrasonic cleaner bath at 40 kHz. Samples were taken every 10 minutes to obtain the lipid release profile.

Lipid Quantification

Lipid concentration was determined using the SPV method with canola oil as the calibration standard. The standard curve was prepared by plotting lipid amount against UV-Vis absorbance and expressed as:

$$y = ax + b$$

where y is absorbance, x is lipid amount, a is the slope, and b is the intercept. The absorbance of the extract at each sampling time was converted into lipid concentration using the calibration equation. The lipid yield was calculated as:

$$Y_L = \frac{m_L}{m_B} \times 100\%$$

where Y_L is the lipid yield, m_L is the mass of extracted lipids, and m_B is the mass of dry biomass.

FINDINGS AND DISCUSSION

Standard Curve Validation

The SPV method was selected because it is practical, relatively fast, and requires only a UV-Vis spectrophotometer. The method is based on the formation of a red-purple complex from the reaction of vanillin with products formed from lipid degradation by concentrated sulfuric acid. The color intensity is related to the lipid amount in the sample. Canola oil was used as the calibration standard and treated using the same procedure as the extraction samples.

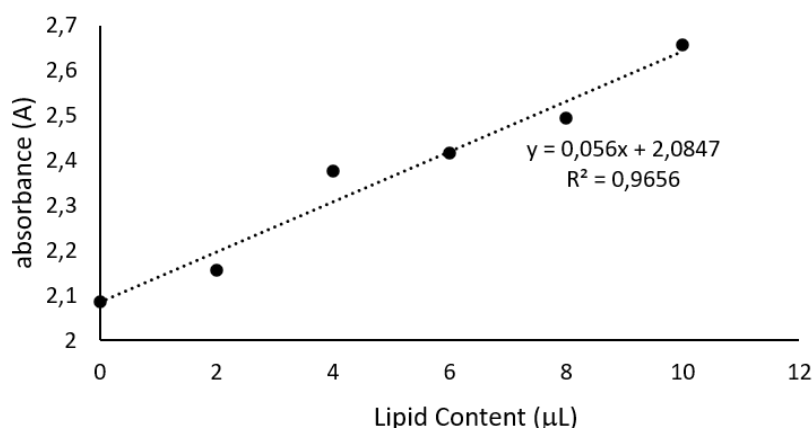


Figure 1. Standard Curve-Relationship between Lipid Amount and UV-Vis Absorbance Using the SPV Method

Figure 1 shows the standard curve for canola oil at 2–10 μL. The linear equation was $y = 0.056x + 2.0847$, with $R^2 = 0.9656$

The correlation was considered adequate for the lipid range obtained in this research, although some deviation was observed. The SPV method has an important limitation because its response can differ between lipid classes. Therefore, the values obtained should be considered relative lipid concentrations rather than absolute lipid contents.

The SPV method was nevertheless suitable for this research because samples were collected every 10 minutes and only a small sample volume was needed. Gravimetric analysis or GC-FAME would provide more specific information, but these methods require additional sample preparation and instrumentation. Therefore, SPV was used to evaluate the extraction profile and compare the pretreatment conditions. Further validation using GC-FAME or gravimetry is still recommended.

Lipid Extraction Without DES Pretreatment

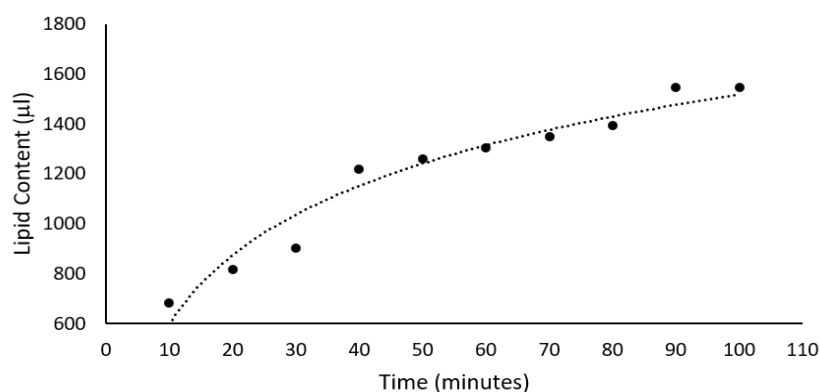


Figure 2. Lipid Extraction Results Without DES Pretreatment

The untreated biomass showed an increasing lipid extraction trend as ultrasound time increased. During the first 30 minutes, the lipid amount increased slowly from around 700 to 900

μL . A sharper increase occurred between 30 and 40 minutes, which may be related to further disruption of the cell wall by ultrasonic cavitation. After 40 minutes, the extraction rate became slower and approached a plateau, reaching approximately 1,546.47 μL at 100 minutes.

The plateau indicates that a portion of the intracellular lipid remained difficult to extract. This is consistent with the role of the rigid cell wall as a mass-transfer barrier. [Ren et al. \(2021\)](#) also reported that microalgal lipid extraction is generally less efficient without an effective cell-disruption step. Therefore, pretreatment is important when the objective is to increase lipid recovery rather than only extending the extraction time.

Lipid Extraction After CCA Pretreatment

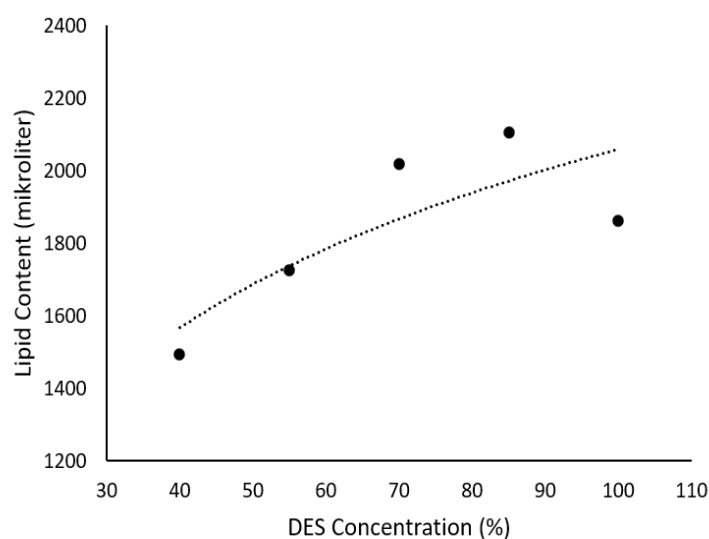


Figure 3. Lipid Extraction Results Using DES CCA Pretreatment

CCA pretreatment increased lipid recovery compared with the untreated biomass, but the effect depended on DES concentration. At 40% CCA, the extracted lipid amount was about 1,493 μL . The relatively low recovery can be associated with the higher water fraction, which reduces the strength of the DES hydrogen-bond network.

The lipid recovery increased at intermediate concentrations and reached the optimum at 85%, with 2,105.18 μL . At this condition, the DES appears to have a suitable balance between fluidity and interaction with the biomass. The solvent can still penetrate the cell-wall structure while maintaining sufficient interaction with the structural components.

At 100% CCA, the lipid recovery decreased to 1,861 μL . The decrease can be related to the higher viscosity of the concentrated DES. High viscosity reduces molecular diffusion and makes solvent penetration more difficult. Similar effects of high-viscosity DES systems on extraction efficiency have been reported by Zhang et al. (2025b). Thus, increasing DES concentration does not automatically increase lipid recovery.

Lipid Extraction After CCU Pretreatment

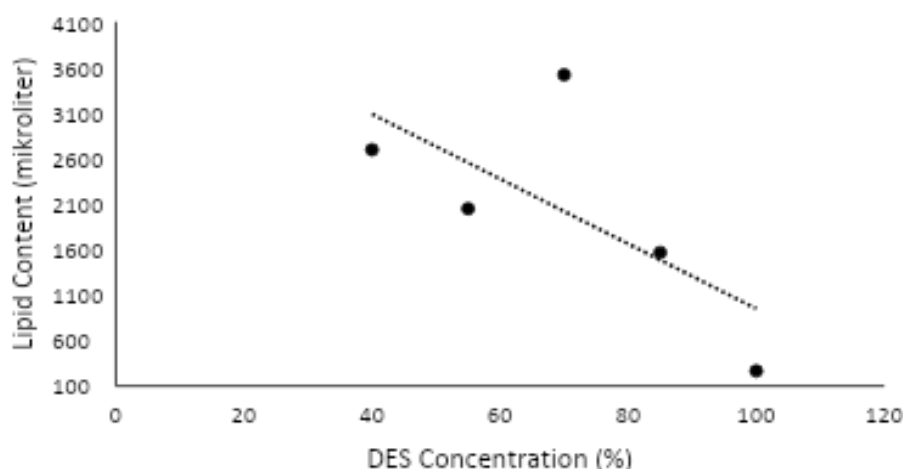


Figure 4. Lipid Extraction Results Using CCU-based DES Pretreatment

CCU showed a different extraction profile from CCA. The lipid amount started from 2,695 μL at 40% concentration and reached the highest value of 3,516.10 μL at 70%. After that, the lipid amount decreased sharply to 1,565 μL at 85% and 264 μL at 100%.

The result indicates that CCU performance is strongly affected by the balance between hydrogen bonding, polarity, water content, and viscosity. At an intermediate concentration, the solvent can penetrate the biomass more effectively while maintaining sufficient interaction with the cell-wall structure. The presence of water can reduce viscosity and improve diffusion, but excessive concentration increases viscosity and reduces molecular mobility. (Zhang et al., 2025a)

The optimum condition at 70% suggests that the CCU system had a more suitable balance between solvent mobility and interaction with the biomass. At higher concentrations, the increase in viscosity becomes more dominant and limits mass transfer. This explains the sharp decrease at 85 and 100%. Therefore, the optimum DES concentration should be determined experimentally rather than assuming that a higher DES concentration will always produce higher extraction.

Comparison of Pretreatment Performance

The maximum lipid amounts obtained were 1,546.47 μL for untreated biomass, 2,105.18 μL for 85% CCA, and 3,516.10 μL for 70% CCU. Compared with the untreated biomass, CCA increased the measured lipid recovery by 36.1%, while CCU increased it by 127.4%. Based on these results, CCU at 70% was the most effective condition among the tested pretreatments.

The better performance of CCU may be related to its interaction with the biomass and the balance between polarity and viscosity at the optimum concentration. However, the mechanism should not be considered proven only from the extraction yield. Structural characterization before and after pretreatment is needed to confirm the changes in the cell wall and the interaction between DES and biomass.

This study establishes that CCA and CCU exhibit distinct concentration-dependent lipid extraction profiles, with optimum recoveries at 85% CCA and 70% CCU. These results demonstrate that DES concentration must be experimentally optimized because increasing concentration does not necessarily enhance lipid recovery, particularly when viscosity limits mass transfer.

CONCLUSION AND FURTHER RESEARCH

Conclusion

This research shows that DES pretreatment can improve lipid recovery from *Chlorella vulgaris* before ultrasound-assisted extraction, with the effectiveness strongly dependent on DES composition and concentration. The optimum CCA condition was 85% (v/v), producing 2,105.18 μL lipid, or 36.1% higher than the untreated biomass. For CCU, the optimum condition was 70% (v/v), producing 3,516.10 μL , or 127.4% higher than the untreated biomass.

This study's contribution is the identification of an experimentally determined DES concentration that can substantially improve lipid recovery without relying solely on prolonged ultrasound treatment. The results demonstrate that increasing DES concentration does not necessarily improve extraction performance. Instead, an appropriate balance between DES–biomass interactions, viscosity, water content, and solvent mobility is required. This finding provides a useful basis for developing DES-assisted microalgal lipid extraction processes and for subsequent process optimization and scale-up.

A key limitation is that lipid recovery was evaluated using the SPV method, which provides a relative lipid response and may exhibit different sensitivities toward different lipid classes. Consequently, the reported values should not be interpreted as absolute total lipid concentrations. In addition, the present study did not directly characterize cell-wall disruption, DES–biomass interactions, or the fatty acid composition of the recovered lipids. The experiments also focused primarily on DES concentration and did not systematically evaluate other important process variables.

Further Research

Further research should validate the optimum 70% CCU condition using gravimetric analysis and GC-FAME to determine total lipid content and fatty acid composition. SEM and FTIR analyses should be performed to clarify morphological and chemical changes associated with DES pretreatment. Optimization should also consider the choline chloride-to-urea molar ratio, pretreatment temperature and time, biomass-to-DES ratio, water content, and ultrasound intensity, preferably using response surface methodology. Quantitative extraction kinetics should be established by determining rate constants, equilibrium yield, and activation energy at different temperatures. Finally, DES recovery, regeneration, and reuse should be assessed, followed by techno-economic and life-cycle assessments to determine the feasibility and sustainability of the optimized process at larger scale.

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